HORMONAL REGULATION OF α-AMYLASE INHIBITOR SYNTHESIS IN GERMINATING BARLEY

by

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The in vivo synthesis of α-amylase and of a bifunctional inhibitor of endogenous α-amylase and subtilisin was studied in embryoless barley half grains treated with abscisic (ABA) and gibberellic (GA₃) acids. Fluorography of newly synthesized proteins and of immunoprecipitated α-amylase and its inhibitor separated by SDS-PAGE showed that GA₃ induces the production of large amounts of α-amylase while the synthesis of other proteins, including the α-amylase inhibitor, is reduced. In contrast, synthesis and/or accumulation of the inhibitor is induced in embryoless half seeds incubated with ABA. These results identify the α-amylase inhibitor as an ABA-induced protein in barley aleurone layers and suggest that it functions as an active mediator of amylase activity during the development and germination of barley seeds.

1. INTRODUCTION

During grain development in barley, several albumin proteins are synthesized in the developing endosperm concomitant with the synthesis of large amounts of prolamin storage proteins (10, 16). One of these soluble seed proteins has recently been identified as an inhibitor of the endogenous barley α-amylase 2 isozyme and of the bacterial protease subtilisin and has been shown by amino acid sequencing to be a member of the soybean trypsin inhibitor (Kunitz) family (11, 18, 23, 24). As with other seed proteins (2), the level of translatable mRNA for this bifunctional inhibitor peaks from 15 to 30 days after anthesis (MUNDY and BRANDT, unpublished data).

During germination, the de novo synthesis of α-amylase, which metabolises the starch stored in the endosperm, can be enhanced in the aleurone layers by the plant hormone gibberellic acid (GA₃) (8, 12). By comparing in vivo and in vitro translation products and by hydridization of α-amylase cDNAs to total germinating barley mRNAs, it has been found that the increase in α-amylase synthesis is due to GA₃-stimulated transcription of genes encoding α-amylase (1, 20). The stimulation of transcription can be blocked by the addition of the plant hormone abscisic acid (ABA) to the tissue, and regulation of α-amylase gene expression by ABA has been suggested to occur either at the transcriptional or

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at the translational level (13, 14, 17, 19). These studies have found that the synthesis of a number of functionally uncharacterized polypeptides of $M_r \sim$ 21, 23, 25, 27, 31 and 36×10^3 is stimulated in barley aleurone layers by ABA. According to MUTHUKRISHNAN et al. (19) the synthesis of a polypeptide of Mr 16 - 21×10^3 is enhanced in aleurone layers by the lysine analogue S-2-aminoethyl-L-cysteine, itself an inhibitor of the transcription of the α-amylase gene. In vitro translation products of Mr ~ 23, 30 and 74×10^3 are formed by mRNA isolated from layers treated with ABA (17).

The present study was undertaken to find out whether the synthesis of the inhibitor of α-amylase 2 (18, 23) is hormonally controlled in aleurone layers of germinating barley. Towards this end, rabbit antibodies raised against α-amylase 2 and its inhibitor have been used to probe the ^{35}S-labelled, newly synthesized polypeptides of embryoless half grains incubated for 8 and 16 hours with ABA and GA$_3$.

2. MATERIALS AND METHODS

2.1. Isolation of α-amylase 2 and its inhibitor and antibody production

The isolation of the major barley malt α-amylase 2 isozyme from an extract of germinated barley (Hordeum vulgare L., cv. Nordal) by glycogen precipitation and β-cycloheptaamylose affinity chromatography and the production of antibodies towards this enzyme preparation have been previously described (7). The α-amylase/subtilisin inhibitor was isolated from a similar barley extract by glycogen precipitation followed by CM-cellulose (Whatman CM-52) chromatography after (18). Rabbits were immunized with 150 μg purified inhibitor×ml^{-1} in 3 dorsal subcutaneous depots of 0.3 ml and tapped after a standard immunization schedule (9). IgGs were purified from the crude antisera by chromatography on protein A-Sepharose 6B. The monospecificities of the IgG preparations were checked by SDS-PAGE of extracts of germinated barley half seeds followed by electrophoretic blotting of the proteins and immunological assay essentially as described in (21).

2.2. Labelling and analysis of proteins synthesized in quartered half seeds

Barley (Hordeum vulgare, L., cv. Nudinka) embryoless, half grains were surface sterilized with 0.2% AgNO$_3$ and imbibed for 3 days on sterile, wet sand. Samples of 20 half seeds were quartered and incubated in 20 ml of solutions containing the hormones (50 μM-ABA and 10 μM-GA$_3$, Sigma). These hormone concentrations have been found appropriate in an in vivo/in vitro study (17) and in a cytochemical study of α-amylase synthesis in whole seeds (8). After 4 and 12 hours the seed portions were placed in fresh solutions. At 6 and 14 hours fresh solutions containing 30 μCi ^{35}S-methionine (Amersham) were added, and the incubations continued for 2 hours. The seed portions were then rinsed in water, frozen in liquid nitrogen, and powdered in a mortar and pestle. The powders were extracted by shaking for 20 min at room temperature in 5 ml 1 mM-CaCl$_2$, centrifuged at 16,000×g for 10 min and the supernatants lyophilized. The samples were dissolved in 50 mM-Tris-HCl, pH 7.5, 0.15 M-NaCl, 2% Triton X-100, and 10 mM-methionine, TCA precipitable counts measured, and aliquots containing equal radioactivity taken for analysis of total in vivo synthesized proteins and for sequential immunoprecipitation with α-amylase antibodies followed by inhibitor antibodies as described in (15). Proteins were separated by SDS-PAGE in a 12.5% gel according to (4) with a sample buffer of 0.2 M-Tris-HCl, pH 8.8, 0.5 M-sucrose, 0.01% Bromphenol blue, 5 mM-Na-EDTA, 1% methionine, 4% SDS, 10 mM-DTT, and 10 mM-iodoacetamide. Gels were prepared for fluorography after (3) and exposed to RP Royal X-omat film (Kodak) at -70 °C for 60 days, and then developed.

3. RESULTS

3.1. Specificities of the antibody preparations

The monospecificity of the α-amylase/subtilisin inhibitor antibody preparation is revealed by the immune-blotting test presented in Figure 1. It appears that the α-amylase 2 antibody preparation contains low amounts of antibodies towards the inhibitor, a contamination which is due to the co-elution of small quantities of
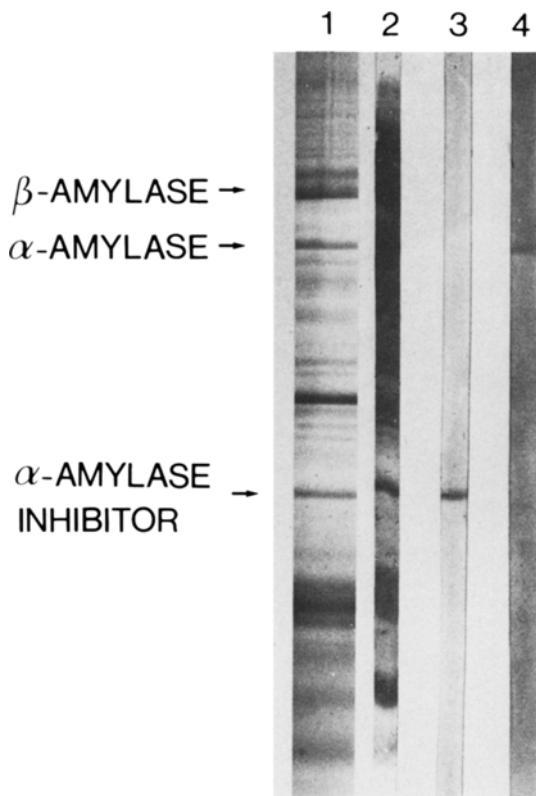
β-AMYLASE →

α-AMYLASE →

α-AMYLASE INHIBITOR →

Figure 1. Immune-blotting assay of proteins in embryoless barley half grains after separation by SDS-PAGE Lanes: 1, proteins in germinated barley half seeds were extracted with sample buffer, separated by SDS-PAGE and stained with Coomassie brilliant blue R-250; 2, proteins transferred electrophoretically in 0.15M-Tris-glycine buffer (pH 8.9), 20% methanol to nitrocellulose paper (Millipore type HAHY) and stained with amido black; 3 and 4, nitrocellulose strips incubated with 0.2 mg×ml^{-1} rabbit antibodies raised against the α-amylase inhibitor and α-amylase, respectively, assayed with peroxidase-labelled swine anti-rabbit antibodies.

inhibitor-enzyme complexes with free α-amylase from the β-cycloheptaamylose affinity column (18).

3.2. Newly synthesized proteins in embryoless half grains

The fluorograph of total proteins synthesized by portions of embryoless half grains after a 16 hours incubation (Figure 2, lanes 1-4) shows patterns similar to those observed by others (14, 17). In GA$_3$ treated tissue (lane 3), the increase in the synthesis of α-amylase ($M_r \sim 42 \times 10^3$) is accompanied by a decrease in the number of other newly synthesized polypeptides. In ABA-treated tissue (lanes 2 and 4) synthesis of α-amylase is markedly decreased while the synthesis of polypeptides with an $M_r \sim 14, 20, 25, 28, 31, 35$ and 50×10^3 appears to be increased compared to both the control and GA$_3$ treatments (lanes 1 and 3). The fluorograph of polypeptides synthesized after 8 hours revealed similar patterns. Especially the synthesis of polypeptides of $M_r \sim 14, 20, 27$ and 35×10^3 appeared to be stimulated by ABA. The amount of α-amylase was not as pronounced at 8 hours as at 16 hours, presumably because the amount of translatable mRNA for α-amylase first reaches a maximum after 10 hours of incubation (14).

3.3. Immunoprecipitation of proteins synthesized in embryoless half grains

Since the embryo and scutellum has been removed prior to incubation of the half grains, it is likely that the synthesis of the proteins studied takes place in the aleurone layers. As judged by the amount of immunoprecipitates, the synthesis of α-amylase is the same upon incubation in the absence and presence of both ABA and GA$_3$ (Figure 2, lanes 5 and 8). The strong enhancement of α-amylase synthesis by GA$_3$ and its inhibition by ABA illustrated in lanes 6 and 7 is in agreement with previous work (14, 17), indicating that the conditions employed in the present experiment are comparable.

Immunoprecipitation with antibodies to the inhibitor shows that very small amounts of inhibitor ($M_r \sim 20,500$) are synthesized in aleurone layers in the absence of the hormones (lane 9). The regulation of synthesis of this polypeptide is the reverse of that for α-amylase; synthesis and/or accumulation of the inhibitor is induced by ABA and inhibited by GA$_3$ (lanes 10 and 11). The induction of the synthesis of the inhibitor by ABA does not appear to be strongly affected by the presence of GA$_3$ in the medium at the levels used in this experiment (lane 12). It should be noted that the apparent co-precipitation of α-
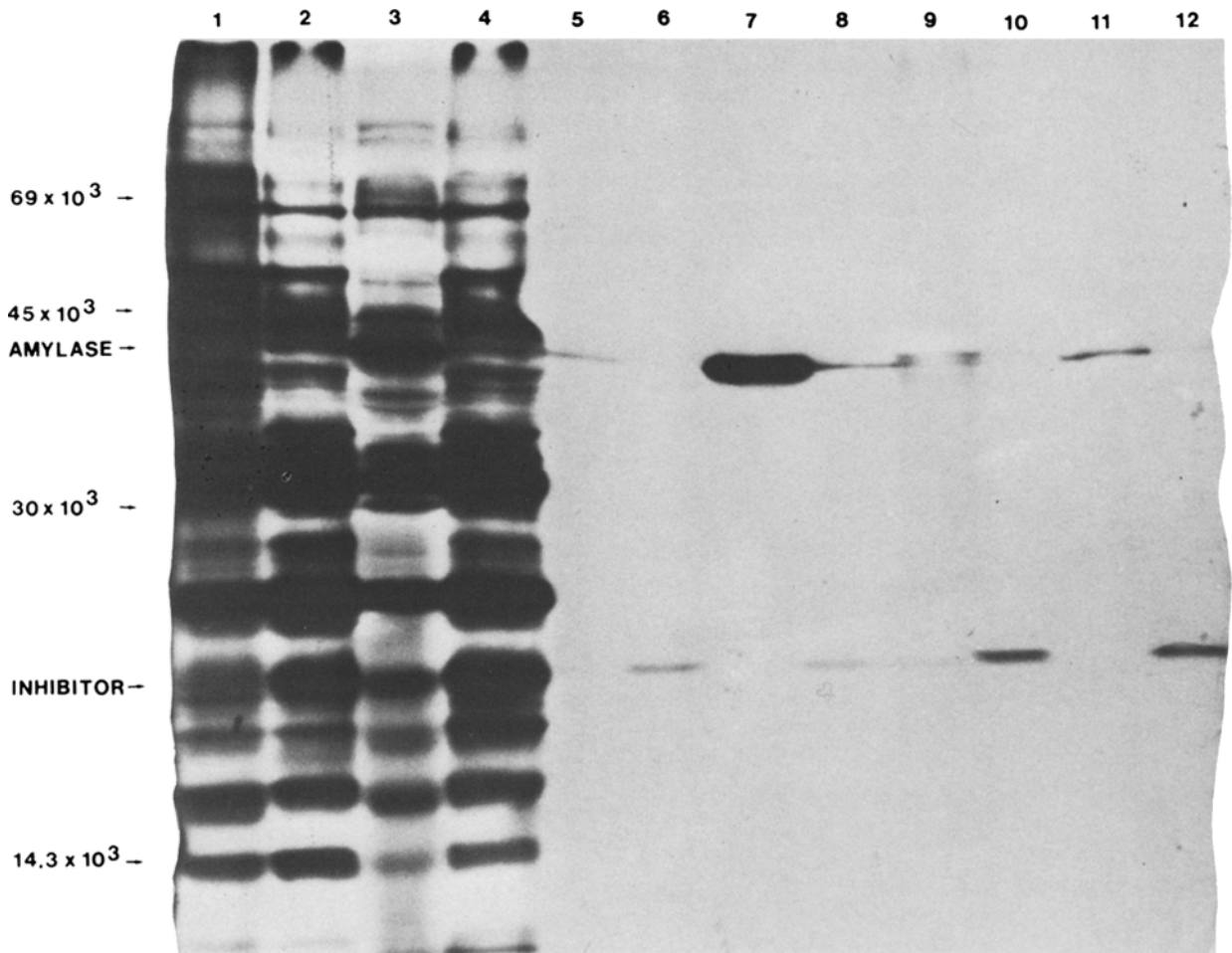
Figure 2. Fluorography of ^{35}S-methionine labelled proteins synthesized in embryoless barley half grains after separation by SDS-PAGE. Half seeds incubated for 16 hours; 1, without hormones; 2, with 50 μM-ABA; 3, with 10 μM-GA$_3$; 4, with both hormones; 5-8, immunoprecipitates of the same extracts with α-amylase 2 antibodies; 9-12, immunoprecipitates of the same extracts with α-amylase inhibitor antibodies.

amylase with the inhibitor (lanes 9, 11, and 12) is due to carry over of α-amylase 2 antibodies from the first immunoprecipitation step (lanes 4-8).

4. DISCUSSION

ABA does not radically alter the pattern of protein synthesis in barley aleurone layers, the physiologically active tissue in nature barley half seeds. Titrations of mRNA with cDNA clones indicate that ABA blocks the synthesis of α-amy-

lase at the transcriptional level (13, 19) while enhancing the synthesis of several proteins of unknown function in isolated aleurone layers and in barley half seeds (14, 17). The results presented here show that the barley α-amylase/subtilisin inhibitor is one of the polypeptides which is synthesized in increased amounts in barley aleurone layers upon treatment with ABA (22). It is not possible to conclude from the present results whether the ABA-induced synthesis of the α-amylase inhibitor is due to an increase in the transcription of genes encoding

the inhibitor, to an increase in the translational efficiency of pre-existing inhibitor mRNAs or to a reduction in turnover of newly synthesized inhibitor molecules. Synthesis of the inhibitor does not occur in embryoless half seeds treated with GA₃, a finding in keeping with the conclusions of others that GA₃ redirects overall transcription to produce large amounts of mRNA coding for α-amylase and a few other polypeptides in the aleurone layers.

The fact that the barley α-amylase/subtilisin inhibitor is synthesized as a seed protein during endosperm development and that its synthesis can be induced in germinating seeds by ABA suggests that it may function in the prevention of precocious germination. Polypeptides normally synthesized during embryogenesis whose synthesis can be induced by ABA during germination have been described in cotton seeds (6) and it will be interesting to study if some of these cotton proteins are hydrolase inhibitors.

Although it is possible that the barley inhibitor is synthesized to inhibit the activity of precociously made α-amylase activity in developing seeds, it could also represent one of many seed proteins controlled by ABA. Work on storage protein synthesis in embryos of Brassica napus points to such a broad response of developing seed tissues to ABA (5). Further work is therefore needed to determine 1) the physiological role of the bifunctional barley inhibitor in regulating amylolytic activity during development and germination, and (2) the molecular mechanism by which ABA induces inhibitor synthesis and/or accumulation.

ACKNOWLEDGEMENTS

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